

SYNERGISTIC COMPOSITIONS FOR THE SELECTIVE CONTROL OF TUMOR TISSUE

Field Of The Invention

This invention is a Divisional of U.S. patent application Serial No. 09/413,449 filed on November 15, 2000 which is, in turn, a Continuation-In-Part of U.S. application serial number 09/446,570 filed on March 27, 2000, now U.S. Patent Number 6,395,720. The invention relates to novel synergistic compositions which selectively control tumor tissue, while healthy tissue is virtually unattacked. The novel compositions are therefore outstandingly suitable for cancer therapy.

Background Of The Invention

Medicaments according to the prior art which are employed in chemotherapy as a rule only achieve partial success, i.e. they do not lead to a final cure. Moreover, the substances employed in the prior art frequently act only in a certain tumor category. A further disadvantage of the currently known chemotherapeutics are their often harmful side effects, as chemotherapeutics can generally have a cytostatic effect on proliferating tissue. The known chemotherapeutics are also unsatisfactory in the control of metastasis formation, and this is one of the main reasons which has until now prevented a decisive outcome in cancer therapy.

The fact that tumor tissue in an extracellular medium has a lowered average pH of about 6.5 to 7.0 and the pH can even fall to 5 on the cancer cell surface, while the pH in the normal tissue and in the blood is approximately 7.2 to 7.5, is known and described, for example in DE-A 44 07 484 and in Tumor Biol., 1994., 15: 304-310. Thus each type of tumor has an intrinsic average intercellular pH, which, for example, in the case of breast tumors is about 6.7 and in the case of colon tumors about 6.9.

In the abovementioned publications, it is disclosed that due to the lowering of the pH range in tumor cells the natural immune defense is blocked, as the body's own defense cells react to cancer target cells with full cytotoxicity only in a slightly basic medium of pH more than 7. DE-A 44 07 484 therefore proposes to bring the acidic external medium of cancer cells to the normal physiological pH level of 7 to 7.5 and thereby to control the cancer cells by means of the body's own immune defense. For this purpose, the acidic external medium of cancer cells is brought to a physiological pH of 7 to 7.5 either by artificial basification measures or by the prevention of the acidification process itself.

The medicaments described in DE-A 44 07 484 admittedly represent an advance in cancer therapy, it would be desirable, however, to have available medicaments which besides the body's own immune defense selectively control tumor cells and thus can be used as relatively low-side-effect chemotherapeutics.

Accordingly, WO 96/30003 proposes to use those compounds for the control of tumor tissue which, at a pH of less than 7, are protonated or release a substance, the protonated compound or the released substance having a more strongly destructive effect on cells than the unprotonated compound or the compound before release of the substance. For these compounds, WO 96/30003 discloses general formulae under which come a multiplicity of chemical compounds. Inter alia, 4-amino-2-hydroxybenzoic acid is mentioned as being effective there. Acetylsalicylic acid is also mentioned as a possible active compound, but this compound is not preferred.

The compounds of WO 96/30003 and also the mixtures generally proposed there of two and more compounds admittedly do already have a good anti-tumor effect, but, as before, there is a need for medicaments which have an improved anti-tumor effect, in particular at pH values of 7.0 or below, in particular in the range from 6.5 to 7.0.

It is therefore an object of the present invention to make available novel medicaments which have a strong cytotoxic effect which is largely selective on tumor tissue, in particular in a pH range from approximately 6.5 to approximately 7.0.

This object is solved by the subject of the patent claims.

The compositions according to the invention in principle act in the same manner as the benzoic acid derivatives disclosed in WO 96/30003. It has been shown, however, that these benzoic acid derivatives, which are known from WO 96/30003, do not have completely satisfactory activity against tumor tissue. Surprisingly, however, individual benzoic acid derivatives as a mixture with one another have a very strongly synergistic action for the destruction of tumor tissue and in a pH range of approximately 7.0 and below cause a virtually complete cell death in tumor tissue.

Summary Of The Invention

The compositions according to the invention are mixtures which contain at least two of the following benzoic acid derivatives or cinnamic acid derivatives, respectively, which act synergistically as a mixture with one another:

- 2-acetoxybenzoic acid (acetylsalicylic acid, Aspirin[®])
- 2-hydroxybenzoic acid
- 2-methoxybenzoic acid
- 2,4-dihydroxybenzoic acid
- 2,4-diacetoxybenzoic acid
- 2-hydroxy-4-aminobenzoic acid
- 2,4-dimethoxybenzoic acid
- 2,6-dihydroxybenzoic acid
- 2,6-dimethoxybenzoic acid
- 2,3,4-trimethoxybenzoic acid
- 2,4,6-trihydroxybenzoic acid
- 2,4,6-trimethoxybenzoic acid
- 5-(2,4-difluoro-phenyl)salicylic acid

α -cyano-3-hydroxycinnamic acid
 α -cyano-4-hydroxycinnamic acid
 α -fluorocinnamic acid
 α -methylcinnamic acid and
 α -acetamidocinnamic acid.

Preferably, the mixture contains at least two of the following benzoic acid derivatives, which act synergistically as a mixture with one another:

2-acetoxybenzoic acid (acetylsalicylic acid, Aspirin[®])
2-methoxybenzoic acid
2,4-diacetoxybenzoic acid
2-hydroxy-4-aminobenzoic acid
2,4-dimethoxybenzoic acid
2,6-dihydroxybenzoic acid
2,6-dimethoxybenzoic acid
2,3,4-trimethoxybenzoic acid
2,4,6-trihydroxybenzoic acid and
2,4,6-trimethoxybenzoic acid

These compounds are known as such, commercially available and can be prepared by a person skilled in the art without problems. They show their particular therapeutic benefits, however, only in the compositions according to the invention.

A synergistic effect is not seen with all mixtures of the abovementioned benzoic acid derivatives. Whether a synergistic action may be present in a mixture can be easily determined by a person skilled in the art taking into account the details below.

Synergistic compositions are, for example, the following mixtures:

2,6-dihydroxybenzoic acid / 2-hydroxy-4-aminobenzoic acid

2,6-dihydroxybenzoic acid / acetylsalicylic acid
 2,6-dihydroxybenzoic acid / 2,4-diacetoxybenzoic acid
 2,6-dihydroxybenzoic acid / 2,4-dimethoxybenzoic acid
 2-hydroxy-4-aminobenzoic acid / acetylsalicylic acid
 2-hydroxy-4-aminobenzoic acid / 2,4-dimethoxybenzoic acid and
 2,4-dimethoxybenzoic acid / 2-acetoxybenzoic acid.

Furthermore, the following mixtures can also be mentioned:

2,4,6-trihydroxybenzoic acid / 2,4-dimethoxybenzoic acid
 2,4,6-trihydroxybenzoic acid / 2,6-dihydroxybenzoic acid
 2,4,6-trimethoxybenzoic acid / 2,6-dihydroxybenzoic acid
 2,4,6-trimethoxybenzoic acid / 2-hydroxy-4-aminobenzoic acid
 2,4,6-trimethoxybenzoic acid / 2,4,6-trihydroxybenzoic acid
 2,4,6-trimethoxybenzoic acid / 2,4-dimethoxybenzoic acid.

As further mixtures can also be mentioned:

2-hydroxy-4-aminobenzoic acid / 5-(2,4-difluorophenyl)-
 salicylic acid
 2-acetoxybenzoic acid / 5-(2,4-difluorophenyl)salicylic acid
 2-acetoxybenzoic acid / α -cyano-3-hydroxycinnamic acid
 5-(2,4-difluorophenyl)salicylic acid / α -cyano-3-hydroxy-
 cinnamic acid and
 2-hydroxy-4-aminobenzoic acid / α -cyano-3-hydroxycinnamic
 acid.

The use of triple combinations of the mixtures is also especially preferred, whereby the following triple combinations have advantageous synergistic properties:

2-hydroxy-4-aminobenzoic acid / 2-acetoxybenzoic acid / 5-
 (2,4-difluorophenyl)salicylic acid
 2-hydroxy-4-aminobenzoic acid / 2-acetoxybenzoic acid / α -
 cyano-3-hydroxycinnamic acid
 2-hydroxy-4-aminobenzoic acid / 5-(2,4-difluorophenyl)-

salicyclic acid / α -cyano-3-hydroxycinnamic acid and 2-acetoxybenzoic acid / 5-(2,4-difluorophenyl)salicyclic acid / α -cyano-3-hydroxycinnamic acid.

In the abovementioned mixtures 2-hydroxybenzoic acid (salicyclic acid) can also be used instead of acetylsalicyclic acid.

An example which may be mentioned of a nonsynergistic composition of the above constituents is a mixture of 2,4-diacetoxybenzoic acid and 2-hydroxy-4-aminobenzoic acid.

On account of their pH sensitivity, the compositions according to the invention are only activated in cancer tumors and metastatic areas and therefore represent an ideal cancer therapeutic. It is also to be particularly emphasized that this novel cancer therapeutic acts generally on all tumor types independently of the specific type of cancer.

Brief Description Of The Drawings

Figures 1-4 show the photometric measurement relationship between the initial pH and wavelength for the cells identified in Tables 1-3;

Figures 4-5 show a recording of the death of the cells in a ELISA(apoptosis kit) with RT112 as a function of pH;

Figure 6 is a graph of the activity of cellular Salicylicacid Combinations Against the Human Pancreas Carcinoma PAXF 736; and

Figure 7 is a graph of Body Weight Changes During Treatment With Salicylicacid Combinations in the Human Pancreas Carcinoma PAXF 736.

Detailed Description Of The Invention

It is assumed that the substance mixtures according to the invention have toxic properties in the protonated state and act as cellular toxins on the tumors.

It is known that the pHs in the extracellular tumor tissue can be lowered again by about 0.5 pH units by inducing acidosis by glucose administration. Such an administration of glucose can likewise take place with the compositions according to the invention.

The compositions according to the invention can contain the active compounds in any desired proportions provided that the synergistic effect still occurs. How the activity of compounds and thus also the presence of a synergistic effect can be detected is described in detail, for example, in WO 96/30003, and in this respect reference can be made to this publication. Reference is further made to the following comparison examples.

The compositions according to the invention preferably contain the two active compounds in the ratio 1:9 to 9:1, particularly preferably in the ratio 2:1 to 1:2 and in particular in the ratio of approximately 1:1.

The synergistic activity of the compositions according to the invention was clearly confirmed by in vitro experiments. In the following, the following short forms are used for the following substance names:

Substance 121 = acetylsalicylic acid (2-acetoxybenzoic acid)

Substance 58a = 2-hydroxy-4-aminobenzoic acid

Substance 132 = 2,4-diacetoxybenzoic acid

Substance 136 = 2,6-dihydroxybenzoic acid

Substance 188 = 2,4-dimethoxybenzoic acid

The experiments according to the invention were carried out as follows:

The measurements were carried out using a "Cell Death Detection ELISA" kit, Cat. No. 1774 425 commercially available from Boehringer (Mannheim). The process instructions are additionally supplied by Boehringer.

The results can be seen from Tables 1 to 3 below. The OD value corresponds to the cell death.

Table 1 (Photometric measurement after 35 minutes)

Compound	Initial pH	OD (405-490 nm)
CAM (800 ng/ml)	pH 6.0	0.818
	pH 6.5	3.037
	pH 7.0	2.725
	pH 7.4	2.416
136 (5 mM)	pH 6.0	0.187
	pH 6.5	0.918
	pH 7.0	0.321
	pH 7.4	0.382
136 (7 mM)	pH 6.0	0.221
	pH 6.5	1.287
	pH 7.0	0.524
	pH 7.4	0.816
Negative control	pH 6.0	0.642
	pH 6.5	0.702
	pH 7.0	0.293
	pH 7.4	0.268
Positive control		3.145

Table 2 (Photometric measurement after 35 minutes)

Compound	Initial pH	OD (405-490 nm)
CAM (160 ng/ml)	pH 6.0	0.344
	pH 6.5	0.711
	pH 7.0	1.220
	pH 7.4	1.923
58a (15 mM)	pH 6.0	0.320
	pH 6.5	0.282
	pH 7.0	0.574
	pH 7.4	1.835
121 (10 mM)	pH 6.0	1.282
	pH 6.5	0.163
	pH 7.0	0.413
	pH 7.4	1.508
132 (10 mM)	pH 6.0	2.856
	pH 6.5	0.183
	pH 7.0	0.088
	pH 7.4	0.502
188 (15 mM)	pH 6.0	0.256
	pH 6.5	0.309
	pH 7.0	0.502
	pH 7.4	1.854
Negative control	pH 6.0	0.674
	pH 6.5	0.648
	pH 7.0	0.327
	pH 7.4	0.322
Positive control		1.940

Table 3 (Photometric measurement after 20 minutes)

Compound	Initial pH	OD (405-490 nm)
CAM (800 ng/ml)	pH 6.0	1.681
	pH 6.5	1.708
	pH 7.0	1.791
	pH 7.4	2.217
136 + 58a (5 mM/10 mM)	pH 6.0	0.206
	pH 6.5	0.745
	pH 7.0	2.036
	pH 7.4	0.793
136 + 121 (5 mM/5 mM)	pH 6.0	0.750
	pH 6.5	0.486
	pH 7.0	2.564
	pH 7.4	0.970
136 + 132 (5 mM/5 mM) 1st experiment	pH 6.0	0.419
	pH 6.5	0.328
	pH 7.0	0.802
	pH 7.4	0.953
136 + 132 (5 mM/5 mM) 2nd experiment	pH 6.0	0.640
	pH 6.5	0.274
	pH 7.0	1.160
	pH 7.4	1.124
136 + 188 (5 mM/10 mM)	pH 6.0	0.211
	pH 6.5	1.728
	pH 7.0	1.426
	pH 7.4	0.804
58a + 121 (10 mM/5 mM)	pH 6.0	0.577
	pH 6.5	0.480
	pH 7.0	2.227
	pH 7.4	1.238
58a + 132 (10 mM/5 mM)	pH 6.0	0.152
	pH 6.5	0.241
	pH 7.0	0.417
	pH 7.4	1.082
58a + 188 (10 mM/5 mM)	pH 6.0	0.251
	pH 6.5	1.698
	pH 7.0	2.939
	pH 7.4	0.915
132 (5 mM)	pH 6.0	0.407
	pH 6.5	0.371
	pH 7.0	0.422
	pH 7.4	0.916
Negative control	pH 6.0	0.883
	pH 6.5	0.794
	pH 7.0	0.330
	pH 7.4	0.579
Positive control		1.219

The results of the experiments are summarized in Figures 1 to 3, which correspond to Tables 1 to 3. In the figures, the cell death is recorded in a ELISA (apoptosis kit) with RT112 as a function of the pH. That is to say the data on the Y axis of

the figures is a measure of the cell death; the pH of the cells is indicated on the X axis. Figure 1 and Figure 2 show experiments in which individual compounds were employed. Figure 3 shows the synergistic effect of the compositions according to the invention compared with the individual compounds. It stands out that at a pH of between 6.5 and 7.0 the compositions according to the invention cause a cell death which is preferably in the vicinity of or above the CAM value, which virtually corresponds to a complete destruction of the tumor tissue.

The apoptosis-inducing substance CAM used in the experiments is camptothecin (CAM value). In the experiments, positive control means that instead of the apoptotic cancer cells induced by the substances a histone-DNA complex with known detection sensitivity is employed for detection, i.e. an artificial apoptosis product is provided. In the experiments, negative control means that the same detection procedure is carried out, but without addition of substances or substance mixtures.

From the comparison experiments it is obvious that certain mixtures of the abovementioned benzoic acid derivatives have a synergistic effect. The mixture of 2,4-diacetoxybenzoic acid and 2-hydroxy-4-aminobenzoic acid does not show any synergistic effect, and the ability of the mixture to destroy tumor cells is not higher than that of individual compounds in the example of 2,4-diacetoxybenzoic acid.

The compositions according to the invention can be formulated in a known manner for medicaments for mammals, preferably man. In the medicaments, the compositions according to the invention are present as a mixture with a pharmaceutical organic or inorganic excipient, which is suitable for enteral or parenteral administrations. The oral administration of the compositions according to the invention by means of tablets, capsules, powders or in liquid form, such as suspensions, in

solution, as an emulsion or as a syrup, is particularly preferred.

In the case of formulation as tablets, customary pharmaceutical excipients such as sodium citrate, lactose, microcrystalline cellulose and starch, lubricants such as anhydrous silicic acid, hydrogenated castor oil, magnesium stearate, sodium lauryl sulfate and talc, as well as binders such as starch paste, glucose, lactose, gum arabic, mannitol, magnesium trisilicate and talc are used. If the compositions according to the invention are to be administered by means of liquids, customary liquid excipients can be used.

A formulation for injections and infusions is likewise preferred, as is known in the field and described in relevant standard works.

The compositions according to the invention can likewise be formulated in a manner known per se as depot formulations or to give medicaments having delayed or sustained release.

The dosage form of the compositions according to the invention depends on the specific composition and further factors and can be determined by a person skilled in the art on the basis of the condition of the patient to be treated, the severity and type of the disease to be treated, possible side effects of the substance mixtures administered, etc.

The dosage of the compositions according to the invention can be determined by a person skilled in the art depending on the specific disease, the patient and other circumstances and is, for example, 50 mg/kg of body weight up to 300 mg/kg of body weight, preferably 100 mg/kg of body weight up to 200 mg/kg of body weight, of the composition according to the invention per day.

It is obvious to the person skilled in the art that the compounds of the compositions according to the invention can

be administered together or in succession at short time intervals such that they still have their synergistic effect. According to the invention, both the simultaneous administration of a suitably formulated substance mixture and the time-shifted or simultaneous administration of the suitably formulated individual constituents of the compositions according to the invention are included, provided that the time intervals between the administration of the individual constituents are not so large that the synergistic effect is lost.

The time interval between the administration of the individually formulated components is generally not more than 24 hours, preferably not more than one hour. Especially preferred is the joined administration of the formulations or the administration of one formulation immediately after the other.

Therefore, the invention also relates to a pharmaceutical pack consisting of two medicaments each of which contains at least one benzoic acid derivative selected from

2-acetoxybenzoic acid (acetylsalicylic acid, Aspirin[®])

2-hydroxybenzoic acid

2-methoxybenzoic acid

2,4-dihydroxybenzoic acid

2,4-diacetoxybenzoic acid

2-hydroxy-4-aminobenzoic acid

2,4-dimethoxybenzoic acid

2,6-dihydroxybenzoic acid

2,6-dimethoxybenzoic acid

2,3,4-trimethoxybenzoic acid

2,4,6-trihydroxybenzoic acid

2,4,6-trimethoxybenzoic acid

5-(2,4-difluoro-phenyl)salicylic acid

α -cyano-3-hydroxycinnamic acid

α -cyano-4-hydroxycinnamic acid

α -fluorocinnamic acid
 α -methylcinnamic acid and
 α -acetamidocinnamic acid.

and, if applicable, a pharmaceutically acceptable carrier or thinner for the joined or timely shifted administration, whereby the pharmaceutical pack contains at least two different benzoic acid derivatives.

The preferred combinations of active agents of the pharmaceutical packs correspond to the abovementioned preferred mixtures of the invention.

Pharmaceutical pack can also be defined as the hint, for example on the package insert of a benzoic acid containing preparation, that this benzoic acid containing preparation should be administered jointly or timely shifted with another benzoic acid containing preparation. As a result, the invention also relates to a pharmaceutical pack as defined above, which is characterized in that the two medicaments are each provided in a separate package, whereby on one package insert of at least one of the packages it is pointed to the joined or timely shifted administration with the other medicament.

The following galenic example illustrates the invention and is not limiting.

Example

A solution of 2-hydroxy-4-aminobenzoic acid in distilled water is prepared, whereby the concentration of the 2-hydroxy-4-aminobenzoic acid is selected in a way that the prepared solution is from isotonic (34 mg/ml 2-hydroxy-4-aminobenzoic acid) to slightly hypertonic (48 mg/ml 2-hydroxy-4-aminobenzoic acid), sterile and pyrogene free. Parallel thereto, a solution of 2-acetoxybenzoic acid obtainable under

the tradename Aspisol is provided. Both solutions are administered to a patient intravenously one after the other, whereby the 2-hydroxy-4-aminobenzoic acid is administered in a dosage of 300 mg/kg body weight and the 2-acetoxybenzoic acid is administered in a dosage of 50 mg/kg body weight.

In particular those compositions are highly active which contain 5-(2,4-difluoro-phenyl)salicylic acid in combination with another salicylic compound and in particular with another salicylic compound as defined earlier. Thus, particularly preferred compositions of the present invention are compositions which contain 5-(2,4-difluoro-phenyl)salicylic acid in combination with one or more compounds selected from the group consisting of

2-acetoxybenzoic acid (acetylsalicylic acid)
 2-hydroxybenzoic acid
 2-methoxybenzoic acid
 2,4-dihydroxybenzoic acid
 2,4-diacetoxybenzoic acid
 2-hydroxy-4-aminobenzoic acid
 2,4-dimethoxybenzoic acid
 2,6-dihydroxybenzoic acid
 2,6-dimethoxybenzoic acid
 2,3,4-trimethoxybenzoic acid
 2,4,6-trihydroxybenzoic acid
 2,4,6-trimethoxybenzoic acid
 salicylic acid.

Among the most preferred combinations of the present application are the following combinations:

5-(2,4-difluoro-phenyl)salicylic acid → acetylsalicylic acid
 5-(2,4-difluoro-phenyl)salicylic acid → 4-aminosalicylic acid
 5-(2,4-difluoro-phenyl)salicylic acid → 2,6-dihydroxybenzoic acid

5-(2,4-difluoro-phenyl)salicylic acid → 2,4,6-trihydroxybenzoic acid

5-(2,4-difluoro-phenyl)salicylic acid → 4-aminosalicylic acid and acetalsalicylic acid.

To avoid any confusion in the following a short table is presented summarizing the abbreviations and chemical names used in the Figures, the claims and the description:

Diflu = 5-(2,4-difluoro-phenyl)salicylic acid (also known as "Diflunisal")

ASA = acetylsalicylic acid = 2-acetoxybenzoic acid

PAS = 4-aminosalicylic acid = 2-hydroxy-4-aminobenzoic acid

HSA = 6-hydroxysalicylic acid = 2,6-dihydroxybenzoic acid

DHSA = 4,6-dihydroxysalicylic acid = 2,4,6-trihydroxybenzoic acid.

The compositions of the present application show excellent activity in tumor therapy and can be used for the treating of all kinds of tumor diseases. The activity has been shown by in vivo and in vitro experiments as explained below.

The antitumor activity of the following salicylate combinations in vivo:

Diflu + PAS (4-Aminosalicylic acid)

Diflu + ASA (Acetyl-salicylic acid)

Diflu + PAS + ASA

Diflu = 5-(2,4-Difluorophenyl)salicylic acid

has been tested in a pancreas human tumor xenograft model, i.e., with human pancreatic tumors transplanted to nude mice. In this model the tumor growth can be observed as volume growth, because the tumor pieces of the pancreas carcinoma PAXF 736 are transplanted beneath the skin of the nude mice. The solutions of the salicylates were injected into the tail vein for 7 consecutive days. The compounds of the combinations

were administered at precise time points in order to obtain optimal synergism and to respect plasma clearance times. The doses applied are:

Diflu:	33 mg/kg/d
PAS:	525 mg/kg/d
ASA:	72 mg/kg/d

in dual combinations and

Diflu:	33 mg/kg/d
PAS:	300 mg/kg/d
ASA:	54 mg/kg/d

in the combination Diflu/PAS/ASA.

The control mice were injected physiological NaCl-solution. After 7 days administration no further injections into the tail vein were possible because of vein injuries.

Within the time of therapy the tumor growth is completely stopped. From there on the tumor starts slowly growing because of incomplete killing of tumor cells in the tumor. The tumor volumes are medium average volumes each taken from 8 tumors, i.e. from 4 mice each bearing 2 tumors. The results are shown in Fig. 6.

The weight control of the mice during the therapy experiment is shown in Fig. 7. It means a control for toxic side effects. As no weight-loss is registered, no side effects by the administration of these salicylates are to be expected.

The anticancer activity of salicylate combinations can also be examined by in vitro experiments with tumor cell lines. As a relevant criterion of toxic effects on cancer cells the proliferation rate can be considered. The proliferation rate effected by salicylate combinations can be measured in dependence of the extracellular pH-milieu. The results of such

an experiment with the adherent tumor cell line RT112 (bladder carcinoma) are shown in Fig. 4. (Details for cell cultures, see Materials & Methods below.)

The effect of salicylate combinations on the proliferation of RT112 tumor cells is quantitated by the measurement of incorporated tritiated thymidine ($[^3\text{H}]\text{-TdR}$) into the cellular DNA of the cancer cells by liquid scintillation counting (LSC). (Method details, see Material & Methods below.)

The experiment demonstrates that at $\text{pH} < 7.0$ the proliferation of the tumor cells is stopped or markedly reduced by salicylate combinations. But it also shows that monoadministration of Diflu, PAS, ASA, HSA and DHSA exhibits no effect or minor effect on the proliferation rate. Diflu/HSA shows comparable or even identical efficiency as Diflu/DHSA does. Both these compound combinations reach the best inhibition of all proliferation.

HSA = 6-Hydroxysalicylic acid

DHSA = 4,6-Dihydroxysalicylic acid.

The toxicity effect of salicylate combinations on cancer cells can also be tested by experiments in which the function of the mitochondrial respiration chain is examined. The respiratory chain rate is determined by the use of a calorimetric assay (XTT-assay) of Boehringer Mannheim. In this assay the non-radioactive dye XTT {sodium 3'-[1-(phenyl-amino-carbonyl)bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate]} is used to evaluate the influence of cytotoxic compounds. The mitochondrial "succinate-tetrazolium reductase" system cleaves the tetrazolium salt XTT to formazan. The amount of this dye is directly correlated to the number of metabolically active cells, as the enzyme system is only active in respiratory and viable cells (method details, see Material & Methods below). Formazan production has been measured as optical density, and the results are shown in Fig. 5. The pH-dependent formazan

production of the control is dramatically decreased by the application of the combinations of the present invention, such as

Diflu + PAS
Diflu + ASA
Diflu + DHSA.

The toxic activity starts at pH 7.2 and is completed at pH 6.9.

Details of the experiments are shown below.

Determination of mitochondrial activity of RT112 using a colorimetric assay (XTT-assay)

The non-radioactive, colorimetric assay system using XTT (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) (BOEHRINGER MANNHEIM) was used to evaluate the influence of cytotoxic molecules on the mitochondrial activity of human tumor cells. The mitochondrial "succinate-tetrazolium reductase" system cleaves the tetrazolium salt XTT to formazan. The amount of this dye is directly correlated to the number of metabolically active cells, as the enzyme system is only active in respirating and viable cells. For this purpose, RT112 cells were harvested, washed twice and resuspended in culture media to yield a density of $2.5 \times 10^5/\text{ml}$. 100 μl /well of the RT112 cell suspension were seeded into 96 well microtiter plates (BECTON DICKINSON (BD), Heidelberg, FRG) and incubated over night at 37°C and 5% CO₂. The supernatant was replaced by 90 μl pH-adjusted culture medium (pH 6.0 - 7.4) and 10 μl of the solution of preferred substances or 100 μl /well pH-adjusted culture medium alone (control). The cells were incubated for various time periods at 37°C and 5% CO₂. Thereafter, cells were washed twice and reincubated in fresh pH-adjusted culture medium. After the incubation period, pH of each well was determined with highly sensitive microelectrodes

before 50 μ l XTT labelling mixture was added. The cells were further incubated for additional 4 h at 37°C and 5% CO₂. The spectrophotometric absorbance of the samples was measured using a microtiter plate (ELISA) reader (DYNATECH, Denkendorf, FRG) at a wavelength of 490 nm (630 nm as reference). Data acquisition and analysis was performed using the BioLinx 2.1 software (DYNATECH).

MATERIALS AND METHODS

Culture of tumor cells

The human bladder tumor cell line RT112 or the human chronic myeloid leukemia cell line K562 were purchased from DKFZ Tumorzell- und datenbank (Institut für experimentelle Pathologie, Heidelberg, FRG) or DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, FRG), respectively. The cells were cultured in HEPES-buffered RPMI 1640 (SIGMA, Deisenhofen, FRG) supplemented with 2 mM L-glutamine (BIOCHROM, Berlin, FRG), 1% NEAE (BIOCHROM), 10% heat-inactivated fetal calf serum (BIOCHROM), 100 U/ml penicillin and 50 μ g/ml streptomycin (BIOCHROM) at 37°C and 5% CO₂. Only mycoplasma-free cell cultures were used; this was frequently tested using a specific detection kit (BOEHRINGER MANNHEIM, Mannheim, FRG).

[³H]-TdR incorporation assay

The influence of cytotoxic molecules on the proliferation of RT112 tumor cells was quantitated by the measurement of the incorporated tritiated thymidine ([³H]-TdR) into the cellular DNA of the tumor cells by liquid scintillation counting (LSC). In brief, RT112 cells were harvested, washed twice and resuspended in culture media (2.5 x 10⁵/ml). 100 μ l/well of the RT112 cell suspension were seeded into 96 well microtiter plates (BECTON DICKINSON) and incubated over night at 37°C and 5% CO₂. The supernatant was replaced by 90 μ l pH-adjusted culture medium (pH 6.0 - 7.4) and 10 μ l of the solution of

preferred substances or 100 μ l/well pH-adjusted culture medium alone (control). The cells were incubated for various time periods at 37°C and 5% CO₂. Thereafter, cells were washed twice, reincubated in fresh pH-adjusted culture medium and pulsed for 24 h by the addition of 23.125 kBq [³H]TdR/well (925 kBq/ml; AMERSHAM, Braunschweig, FRG). After freezing and thawing, radioactive DNA of the cultures were transferred to glass fiber filters with an automatic cell harvester (PHARMACIA). The incorporated radioactivity was measured in a liquid scintillation counter (PHARMACIA).

Preferably, the compositions of the present invention contain 5-(2,4-difluoro-phenyl)salicylic acid and one other salicylic compound or two other salicylic compounds. Thus the medicaments of the present invention contain preferably two or three pharmaceutically active ingredients.